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THE EFFECT ON THE PROTOPLASM OF NITELLA OF VARIOUS CHEMICAL SUBSTANCES AND OF MICRO-ORGANISMS INTRODUCED INTO THE CAVITY OF THE LIVING CELL.*

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Many experiments have been conducted to test the effect on unicellular plants and animals of various substances introduced into the fluid surrounding the cells. In the experiments described in this paper, the test substances were introduced by means of very fine glass capillary pipettes directly into the vacuole or protoplasm of the living cell. By the use of this method the observer may note the effect of the substances when brought into immediate contact with the protoplasm or when introduced into the vacuole, where the only barrier to diffusion into the protoplasm is the protoplasmic layer immediately lining the vacuole. Further, one may test the local effect on the part of the cell into which the substance is injected. The results differ materially from those obtained by applying the chemical outside of the wall, especially as regards the local effects observed.

The technic employed has been described in a previous paper.[1] In this method the cell wall is pierced with pipettes which may be made so small that the lumen scarcely exceeds one micromillimeter in diameter. Such a pipette may be introduced into the relatively large cells of Nitella with little or no injury to the cell. The expansion of mercury behind the substance to be injected furnishes the force necessary for overcoming the cell pressure, and a device for applying heat or cold to this mercury serves to approximately regulate the size of the dose.

Nitella was chosen as a test cell because of its relatively large size, the transparency of its wall, especially in the rhizoids and the portion of the main filament next to the rhizoids, and because of the rapid movement of its protoplasm, which gives the observer a

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[1] *Jour. Infect. Dis.*, 1911, 8, p. 348.

criterion of the effect of the substances introduced. The size of the cells varied from 0.5 mm. in diameter and 50 mm. in length to those 0.2 mm. or less in diameter and of proportional length. The term "cell" is here used for the cornocytc consisting of many nuclei in a common cytoplasm.

The size of the dose was only approximately measured. It varied from a globule of liquid two or three micromillimeters in diameter to quantities approximating $\frac{1}{10}$ of the total volume of the cell. In the table below the expression "small" refers to doses a few micromillimeters in diameter; and the "large" doses as a rule exceed $\frac{1}{100}$ of the volume of the cell. These are only rough estimates but may give some idea of the amounts injected.

It was found that fairly large doses of water, physiological salt solution, or broth had little or no effect on the cells, at most causing only a temporary slowing of the movement of the protoplasm. Very large doses of any substance, suddenly introduced, sometimes caused the death of the cell, possibly as much through mechanical insult as through any purely chemical action. As a rule the action of the chemical injected was at first only local, and in most cases the effect of smaller doses was restricted to local injury.

In many cases there occurred the death or paralysis of only a small portion of protoplasm in immediate contact with the substance injected. There was often in these cases a temporary heaping of protoplasm about the point of injection with a subsequent carrying away of the injured protoplasm and a return of the cell to normal flow. When the toxic effect was a little stronger, enough destruction of protoplasm was effected to form a plug of dead protoplasm often sufficiently large and compact to divide the cell into two parts, each part living and possessing its individual mass of rotating protoplasm. This plug sometimes persisted for hours, and in one case finally disintegrated, leaving the cell normal, except for a mass of dead protoplasm at the point of inoculation, and some fragments of dead protoplasm floating in the protoplasmic current. Finally, with larger doses or stronger poisons, not only local destruction of protoplasm occurred, but cessation of movement in the entire cell soon followed. Unless overwhelming doses were employed, even so powerful a cell poison as bichloride of mercury did not instantly kill the cell, but a local necrosis was followed some minutes later by the stoppage of the protoplasm of the entire cell. The local death of the protoplasm prevents the poison from being quickly carried by the protoplasmic stream to other parts of the cell. The killed protoplasm in the immediate neighborhood of the point of injection usually became less transparent and took on a light brownish color. It sometimes became more vacuolated.

In these experiments it was, of course, necessary to prevent the complication of results by the accidental ejection of the chemical tested outside as well as inside of the cell. To guard against this possibility enough time was usually allowed after the insertion of the pipette to make sure that the flow of the protoplasm was normal before injecting the dose. In the case of strong poisons the tip of the pipette was often washed in water before inserting it into the cell. In several experiments other cells lay near enough to the one tested to serve as controls as regards the presence of the chemical outside of the cell.

Below is given a table in which the chemical substance, the approximate dose, the number of tests, and the effect on the cell are given. Substances are arranged in the table approximately in the order of their toxicity for the cell. The permanent stoppage of protoplasmic movement throughout the entire cell was taken as an indication of the death of the cell. Unless otherwise stated, solutions were made with distilled water.

In reviewing the results of these injections it is noteworthy that strychnine, alcohol, arsenic, and quinine had comparatively slight effect on the cell protoplasm. Saturated solution of copper sulphate in small doses may be introduced into the vacuole of the cell with little effect. Since the protoplasmic layer immediately lining the vacuole opposes some resistance to substances entering from the vacuole, the effect of a substance inoculated directly into a vacuole may be compared with that of a high dilution outside of the cell. Chloroform and ether exerted a powerful local effect, but not so great a general effect as bichloride of mercury or osmic acid. It is to be noted that the amount of chloroform and ether injected was exceedingly small. A tiny globule was forced out

TABLE 1.

Substance Injected	Concentration	Size of Dose	Number of Tests	Effect on Cell of Nitella
Bichloride of mercury..	Saturated solution	Medium	1 2	Local death of protoplasm followed by death of entire cell in about 10 minutes.
		Small	2	In one case plug of dead protoplasm with living protoplasm on either side. In other case death of entire cell.
Bichloride of mercury..	Crystals ground in olive oil	Large	2	In one case early death of cell. In other case cell survived about 3 hrs. Apparently a slow dissolving out of mercury from the oil.
Osmic acid	Saturated solution	Medium	2	Instant local destruction of protoplasm followed in a few minutes by death of the entire cell.
Potassium hydroxide...	50 per cent	Small		Action like that of saturated solution of bichloride of mercury.
Copper sulphate.......	Saturated solution	Large and small	11	Very small doses tolerated. Larger doses followed by local necrosis of protoplasm and later by death of entire cell.
Formaldehyde........	About 4 per cent	Small	1	Local death of protoplasm with formation of plug of protoplasm dividing cell into two living parts.
Formaldehyde........	40 per cent	Small	1	Local death of protoplasm followed by death of entire cell in about 10 minutes.
Chloroform...........	Undiluted	Very small	2	Plugs of dead protoplasm formed with living cell segments on either side. In one case the plug after several hours disintegrated and segments were united.
Chloroform...........	1 per cent	Varying	4	Very small doses followed by local necrosis only. Large doses, local death followed in a few minutes by the death of entire cell.
Ether................	Undiluted	Small	2	Action much like chloroform but less poisonous.
		Large	1	Local death soon followed by death of whole cell.
Alcohol..............	Absolute	Varying	7	Only large doses caused much local death of protoplasm. Small doses effected no apparent harm.
Alcohol..............	95 per cent	Large	5	Little toxic effect.
Arsenic trioxide	Saturated solution	Small and large	3	Little effect of small doses. Larger cause some local destruction of protoplasm.
Fowler's solution......	....	Varying	12	Little if any effect of large doses.
Sulphate of quinine....	Saturated solution	Varying	3	Local death follows large doses. Toxic effect small.
Sulphate of strychnine.	1/500 to 1/10,000	Varying	10	No effect.
Sulphate of strychnine.	Saturated solution	Large	4	Apparently no effect.
Sodium chloride.......	Saturated solution	Medium	..	Slight local destruction of protoplasm, no stoppage of flow.
Sodium chloride.......	0.85 per cent	Large	..	No effect.
Mercury metallic......	....	Globules minute to about $\frac{1}{5}$ diameter of cell	Many	No effect unless large enough globules to cause mechanical injury.
Methylene blue	Nearly saturated	Very large	Many	Very little or no poisonous action.
Water...............	....	Varying	Many	No effect.

into contact with the protoplasm, and, after a minute or so of contact, was withdrawn into the pipette again; so the cell received only the minute quantity that could diffuse from the globule in that very short time. Yet this amount was sufficient to cause the death of a considerable mass of protoplasm. Bichloride of mercury may be injected in small enough dose to cause only local death of the cell.

In some experiments a dose of methylene blue was injected immediately after some protoplasm-destroying agent. It was found that there was no immediate deep staining of the protoplasm, but at most only a stain of the protoplasm that was in immediate contact with the fixative. From this it seems likely that the mere stoppage of protoplasm does not immediately result in its death, or at most does not bring it at once to the stage when it readily takes up dyes. No attempt was made to ascertain if a cell may be rendered tolerant to a poison by small repeated doses. It seemed that this might be more conveniently and accurately done by applying solutions from the outside, though it might be worthy of an experiment to compare the action of substances thus applied with the action when brought directly into the cell cavity.

Some of this work was done while the technic was in the process of evolution and it is recognized that the work lacks definiteness in respect to measurement of the size of the dose, a thing which could be done much more accurately at the present stage of the development of the technic. But it has seemed to the writer that a new province of experimentation has been opened by this method and that this paper may be worth while at least as an introduction to this field of research.

II. THE INFECTION OF LIVING CELLS BY MEANS OF MICROORGANISMS INTRODUCED WITHIN THE CELL CAVITY

The most of this work[1] has been done on plant cells, though some experiments were carried out with the larva of the gnat, *Chironymus*. Aquatic plant cells of large size were for the most part employed, Nitella and Vaucheria of the green plants, and the fish mould genera, Saprolegnia, Achlya, and Dictyuchus of the fungi. These cells were chosen because of their relatively large size, their transparency, and the active circulation or rotation of their protoplasm which gives the observer some criterion of the condition of the cell.

The microorganisms inoculated were vegetating bacteria, spores of bacteria, yeast plants, and spores of fungi. The dose varied from several organisms to hundreds. As a rule no immediate injury to the cell inoculated could be noted following the injection of the dose, even when relatively large quantities of well grown broth cultures were used.

During inoculation the cells were kept in shallow hanging drops in water, and after inoculation were transferred to watch glasses, or kept in moist chambers in hanging drops. Uninoculated cells kept in the same condition served as controls.

The prevention of injury to the cells pierced and the ease with which the wall may be penetrated depend much on the size and character of the point of the pipette. With very fine pipettes, sufficiently large for injecting small bacteria, the cell wall of Nitella or of the fish moulds may be pierced with no disturbance of the flow of the protoplasm. Larger bacteria, yeasts, and the spores of fungi require pipettes of larger openings. Such pipettes sometimes cause loss of protoplasm with a temporary checking of the flow on withdrawal of the pipette after inoculation, but unless the opening made is very large or the wall torn in piercing, it is quickly closed by protoplasm and no lasting injury to the wall results. In the larger Nitella cells some difficulty may be met with in piercing the tough walls with the larger pipettes. If the end of the pipette is broken off slantwise instead of square across, it enters more easily; and if the tip be placed against the wall, some pressure applied, and the mechanical stage moved back and forth, a boring action is obtained which aids much in penetrating the cell.

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[1] Later work with this technic has emphasized the importance of using the abruptly tapering pipette points described in a previous paper. The extension of a part of the glass tube below the loop as described in Fig. 1, *n* (see *loc. cit.*), has been found to be unnecessary. Further, it will perhaps be more convenient for most workers to hold the pipette in the right hand and the forceps in the left in drawing the fine point of the pipette.

When the larger pipette is withdrawn there tends to be an outrush of cell contents owing to the high cell pressure. This outflow may carry with it the material inoculated, or, if the opening is large, may result in too great a loss of contents. In the fish moulds the pipette may be left in the cell until a mass of protoplasm has formed over the tip. The pipette may then be withdrawn without a particle of loss of contents. In case of cells with rapidly rotating contents, as Nitella, the dose injected is soon swept so far away from the point of inoculation that there is little danger of its loss on the withdrawal of the pipette. Considerable loss of contents may be borne by the cells of this plant without apparent injury.

The outflow of contents when a small opening is made in the cell continues until the opening is plugged. This prevents the introduction of bacteria from the surrounding medium or from any source except from out of the pipette. In no case in the experiments described here has an accidental contamination been noted. As controls many cells were pierced but not inoculated. These remained in good condition, in some cases for eight or more days after being pierced.

When pipettes of large opening have to be used, there is sometimes danger of the retreat of a part of the dose injected into the pipette when the pressure is stopped. This may be avoided by regulating the degree of contraction of the mercury in the pressure apparatus. When the tip of the pipette is in a mass of protoplasm, the viscid protoplasm will often act as a valve, closing the mouth of the pipette when pressure is diminished.

In the work described here something over fifty inoculations were made in which bacteria were seen to multiply in the still living cell. No cell was classed as living unless movement of protoplasm could be distinctly made out.

The list of cells inoculated and organisms injected are as follows:

Cell	Organism	Number of Tests Made
Nitella	B. prodigiosus	8
Vaucheria	B. prodigiosus	1
Nitella	B. typhosus	5
Nitella	B. subtilis, rods	3
Nitella	B. subtilis, spores	1
Nitella	Sporotrichum	1
Nitella	Yeast	4
Saprolegnia	Water bacteria	11
Saprolegnia	B. typhosus	2
Achlya	B. typhosus	7
Achlya	B. prodigiosus	1
Dictyuchus	Water bacteria	3
Dictyuchus	B. coli communis	2

It was not always easy to identify the different genera of the fish mould before fruit was formed; but as all behaved nearly alike, an exact botanical classification may not be so important. The Nitella cells chosen were for the most part the more transparent cells found just above the insertion of the rhizoids.

The results of the inoculation of bacteria into both the fungi and the Nitella are so similar that it is hardly necessary to describe each combination in detail. In nearly every case the bacteria grew luxuriantly in the cells, apparently finding there a good medium for growth. Mobile forms generally showed a high degree of motility in the infected cell. In a few cases bacteria apparently living failed to grow, but this might occasionally happen in the transfer of a small number of individuals to a very different medium in a culture tube. As might be expected, the larger the dose the more surely and quickly the cell became infected. Infection followed the introduction of both vegetative forms and spores. In a Nitella filament inoculated with the spores of *B. subtilis* germination of spores was delayed nearly two days at a room temperature of about 26° C. as compared with a control inoculation in broth.

As a rule there was no evidence of a harmful action of the protoplasm of either the fungi or of the green plants on bacteria. Both *B. typhosus* and *B. prodigiosus* swam about freely in the rapidly rotating protoplasm of Nitella. Their movements with respect to the flowing protoplasm showed that many of them were actually in the protoplasm and not in the vacuole of the cell. They would start upstream in the current of protoplasm, succeed in stemming it for a time, finally yielding and being carried down or forced to take some different direction. Their movements seemed to be embarrassed only by the rapidity of the current and the viscidity of the medium, not by any bactericidal power inherent in it. The bacteria apparently meet with no resistance in passing from the cell vacuole into the protoplasmic layer. No case was observed of bacteria passing from one living cell to another, although in many experiments living uninoculated cells immediately adjoined infected cells in the same chain.

In order to further test the effect of protoplasm on bacteria, two Saprolegnia filaments were inoculated with typhoid bacilli, kept at room temperature until the typhoid had well started, then transferred to a refrigerator temperature of about 12° C. At this temperature typhoid bacilli multiply very slowly, if at all, while the fish moulds grow well; so that any tendency on the part of the Saprolegnia protoplasm to destroy the bacteria would be favored. It was observed that the typhoid bacilli multiplied little, but remained actively motile and healthy in the vacuole of the fungus cell for five or six days. A cell of Nitella was also inoculated with typhoid bacilli, left at room temperature of 21°–22° C. for 20 hours until the bacilli were well started, and then transferred to the refrigerator for 74 hours. On bringing the filament to a temperature of 24°–25° C. the bacilli multiplied freely. The Nitella protoplasm continued to rotate actively at refrigerator temperature. In this case, too, the protoplasm did not destroy the bacilli, even though these were at a temperature relatively more unfavorable to them.

As to the effect of the bacteria on the plant cells, the green plants showed the least resistance to infection, generally dying within 12 to 20 hours at room temperature after the bacteria became numerous in the cells. The fish moulds showed rather more resistance, possibly because of the fact that in their natural environment they are usually surrounded by many bacteria and must have become somewhat accustomed to their products. In one case, Dictyuchus inoculated with *B. coli communis*, the cell survived 58 hours in the presence of numerous bacteria in the vacuole. The temperature in this experiment ranged from 20°–27° C. It is noteworthy that the plant cells could live many hours with rapidly circulating protoplasm, when bacteria were so numerous in the vacuole of the cells as to form a thick emulsion. Apparently the toxic effect of the bacteria on the protoplasm is relatively slight; and the injury to the cells results largely from parasitism. One must take into consideration the fact that if toxin is produced it would not remain long in contact with the cell protoplasm, since it would readily diffuse out from filaments wholly immersed in water.

As a rule, the protoplasmic layer became thinner and thinner in the infected cells until its motion ceased. Plasmolysis and the death of the cell followed soon after cessation of motion of the protoplasm.

The fish moulds showed some reaction to infection by walling off the more infected portions by partitions in the filament. In other cases the infected cell transformed itself into a sporangium containing bacteria-free spores. In one cell a mass of protoplasm much larger than a spore was segregated off from the bacteria. In another case a bacillus, apparently motile, was included within a spore. Sometimes bacteria-free branches grew out from the protoplasmic layer lining the infected cells. The Nitella and Vaucheria inoculated showed no such reactions to infection.

In a filament of Achlya infected with slowly growing filamentous typhoid bacilli the protoplasm of the infected cell put out tongue-like processes toward the bacilli, suggesting an attempt at phagocytic action. No distinct engulfing of bacteria could be made out, so there may have been no relation between the form of the protoplasmic tongues and the bacteria. From a Nitella cell, the cell still living and containing numerous prodigiosus bacilli, some bacilli were withdrawn by means of the pipette, and sown on agar. No growth occurred. Though a hundred or so of the bacteria were transferred it may be that not enough were taken to give a fair test of viability. The bacteria in this case were non-motile in the cell.

In summary it appears that these plants depend on the barrier afforded by their walls, or, in case of a break in the wall, by protoplasmic membranes, to protect themselves against infection; and their protoplasm has very slight if any antibacterial power. On the other hand their protoplasm opposes a marked resistance to the action of bacterial products, a characteristic perhaps acquired from the frequent exposure of these cells to decaying substances in their natural habitat. In the presence of a strong cell pressure alone these cells have a protection against introduction of bacteria through some small opening, a protection not shared by plants or animals consisting of masses of cells whose intercellular spaces offer a breeding place for bacteria.

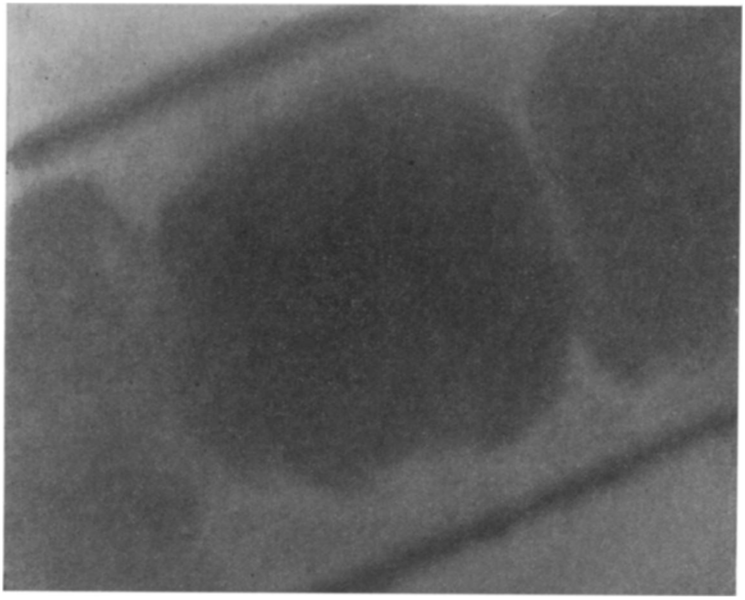

FIG. 1.—A living cell of Nitella containing a large colony of yeast, the darker body in the center, with a mass of protoplasm on either side. The striated appearance at the margins of the cell shows where the protoplasmic stream was most rapid. (Magnification ×160.)

There was but one successful inoculation of a cell with fungus spores, that of Nitella with spores of a Sporotrichum obtained from lesions in the human subject. Small mycelia, at first movable in the rotating protoplasm, developed on the second day. Later the mycelia increased in size and became fixed in the cell. The cell survived about four days, and during this time no spores formed on the fungus.

Possibly the most remarkable results in the whole series were obtained in Nitella inoculated with yeast cells. Four cells were successfully infected with a pure culture of wild yeast obtained from rancid butter. The yeast cells multiplied rapidly in the living protoplasm and vacuole of the cell at room temperature and more slowly at refrigerator temperature. Finally the cells became so closely packed with yeast cells that the contents of the vacuole resembled a thick emulsion where the cells were so numerous as to be practically in contact with one another. In one cell (see Fig. 1) there was at first apparently a single group of yeast plants in the cell. This grew to form a mass with a diameter nearly as great as that of the Nitella, and with edges somewhat rounded by its rolling movement in the protoplasm. Of the four cells one survived six days, one seven days, one eight days, and one ten days. The greater part of this time the plants were kept at refrigerator temperature. The Nitella cells poorly supplied with chlorophyll, and kept in the dark most of the time, could elaborate little food; and it is remarkable that they could harbor such a mass of foreign organisms for so long a time.

SUMMARY.

1. Bacteria, yeasts, and Sporotrichum find a good medium for growth in the interior of certain living plant cells.
2. There is no evidence of an antibacterial property of the protoplasm of these plants.
3. The protoplasm of these cells shows a marked resistance to the products of bacteria and yeasts, and the death of the cell seems due to parasitism rather than to toxins.

The experiments with animal cells thus far are few. Several successful inoculations of the partially transparent larva of the gnat, *Chironymus*, may be mentioned. These larvae were only about five mm. long, and the difficulty of inoculation was increased by the activity of the animal and the toughness of the skin. Larvae were infected with *B. prodigiosus*, the bacteria multiplying in the body cavity but not penetrating the intestine during life. These animals showed a remarkable resistance to the action of bacteria. One larva lived 46 hours with *B. prodigiosus* swarming in the body cavity. Controls showed that the mere piercing of the skin is not necessarily followed by infection. Bacteria recovered from the body of an infected animal proved to be the species inoculated.